High Resolution Imaging of TGFβ3 Treated Human Keratinocyte via a Newly Developed Widefield Surface Plasmon Resonance Microscope

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Abstract— **In this study high resolution imaging of human adult (HaCaTs) Keratinocytes cells using the newly developed Widefield Surface Plasmon Resonance (WSPR) system will be discussed. Surface Plasmon Resonance (SPR) occurs at the interface between a dielectric and a thin conducting layer when p-polarized light strikes at a specific angle thus excites free electrons and generates surface plasmon electromagnetic wave. The SPR excitation angle can be changed by the binding of bio-molecular species to the metallised layer, and is directly proportional to the refractive index and thickness of that molecular species. Our WSPR system provide high lateral resolution imaging close to 500 nanometers [1] and was used to investigate cell surface interactions under two different culture conditions: HaCaTs cultured on SPR substrate with Transforming Growth Factor β3 (TGFβ3) *(50ng/ml)* [2] and without TGFβ3. In less than 24 hours, HaCaTs cultured in the presence of TGFβ3 showed enhanced division and motility along with decreased cell attachment as compared with cells maintained in TGFβ3 free media. It is to be noted that cellular signaling by TGFβ3 is very important for enhancing tissue development in wound repair and that this study for the first time enabled optical interrogation of cell surface interface without the need for Immunostaining.**

Keywords— **Surface plasmons, TGFβ3, HaCaTs, Cell attachments, High resolution imaging .**

I. Introduction

Surface Plasmons (SPs) resonance based imaging is a relatively new field [3] that has a potentially broad range of applications in biological imaging. The pioneering work of E. Kretschmann and H. Raether in 1960s indicated that attenuated total reflectance (ATR) prism based on SPs excitation could be induced in a configuration known as the Kretschmann configuration [4]. In this configuration *p*-polarized light striking the metallic coated surface from a region of high refractive index at a specific angle θ_p will resonantly excite the electrons in the metallic film [5]. This resonance will propagate along the conductor, giving rise to a surface plasmon wave. The SPR angle θ_p is changed when bio-molecular species binds to the coated metal layer and this change in angle enables the thickness of that molecular species to be determined [6]. Surface plasmon microscopes have a wide range of uses, such as chemical sensors, biomolecule and biological binding/attachment sensors, and in any areas where sensitivity to minute changes in the properties of an interface is required. Standard Kretschmann based SP techniques are extremely powerful, but they lack the spatial resolution required to image highly localized regions at micron to submicron scales [3]. We have developed a new WSPR microscope capable of high lateral resolution imaging close to one micron. This system uses a high numerical aperture 1.45, oil immersion objective lens, which allows *p*-polarized light to be applied to a metallised glass slide at an angle capable of exciting surface plasmons [7, 8]. In this system we combined widefield phase confocal microscopy [9] with high resolution scanning SPR microscopy [7] where the imaging in the WSPR microscope is achieved by disruption of coherence using a rotating diffuser conjugated with the back focal plane [3, 8]. When stationary the diffuser illuminates the sample with a speckled pattern, but on rotation this speckled pattern is averaged enabling the generation of a wide field image. At present, various system are available as means for investigating the cell surface interface, such as Atomic Force Microscope (AFM) [10], Quartz Crystal Microbalance, Scanning Force Microscopy, Scanning Nearfield Optical Microsope (SNOM), Field-Emission Secondary Electron Microscopy (FESEM), and various immunostaining methods combined with confocal microscopy [11]. Each of these systems enables high resolution imaging, but do not readily enable the imaging of interfacial interactions. Thus we aim to demonstrate that the new WSPR can be used to image the cell surface interface and interrogate changes at the interface induced by the potent cytokine TGFβ3. TGF-β, is a chemo-

tactic stimulant in fibroblasts where it enhances fibroplasias and the production of extracellular matrix (ECM) components such as collagen and elastin whilst suppressing the ECM breakdown. Thus, TGF-β is an important component in the process of wound healing and is produced naturally by cells such as platelets, macrophages and endothelial cells. There are various isoforms of TGF-β, (TGF-β1, TGF-β2, TGF-β3, TGF-β4 and TGF-β5) and these induce different cellular responses via the same suite of membrane receptors.

II. Materials and methods

Cell Culture: HaCaTs cell lines were cultured in small standard Plug seal cap 25cm^2 culture flask with ratio 1:5 ml cell suspension in Rosewell Park Memorial Institute media (RPMI, SIGMA). Cells were incubated at 37ºC and were split upon reaching confluence, usually every 4-5 days.

SPR Substrate Preparation: Two glass cover slips (0.18mm thick), coated with 50 nm Gold/1 nm Chromium were plated with HaCaTs cells for 24 hours in two conditions i.e. HaCaTs cultured on SPR substrate with TGFβ3 *(50ng/ml)* and without TGFβ3. After 24 hours, the cells were then fixed with 0.1% Glutaraldehyde solution in Hank's Balanced Salt Solution (HBSS, SIGMA) for 5 minutes and dehydrated in serial alcohol. The SPR substrate were imaged with a Ziess Axioplan 2 microscope using a long working distance 40x objective under Differential Interference Contrast (DIC) imaging conditions. The substrates were then mounted in the sample holder of the WSPR microscope and imaged further.

Trypsinisation Process: To verify the effects of TGFβ3 on HaCaTs cell attachment to the surface, HaCaTs cells were cultured in two conditions, with TGFβ3 at 50ng/ml and without TGFβ3 for 6 days in small standard Plug seal cap 25cm^2 culture flask and trypsinised (1ml of 0.25% Trypsin/EDTA were applied to cover the whole surface area of the 25cm^2 culture flask) from the surface upon reaching confluence. The degree of cell attachment was then determined by measuring via time lapse microscopy (1 frame every 20 seconds for 7 minutes) the time required for trypsinisation to induce the cells to take on a rounded phase bright morphology.

III. Results and discussion

In this experiment we imaged human keratinocytes. These cells attach strongly to one another and also to the culture substrate. Imaging with the WSPR system showed that HaCaTs cells attach to the surface via concentrically arranged, high contrast band like structure, with the highest contrast band like components being localized at the cell perimeter and in the lamellapodia (Figs 1e, 1f). However, when HaCaTs cells treated with TGFβ3 were imaged with WSPR system, significant differences in cell morphology were seen at the cell surface interface (Figs 1g, 1h). After treatment with TGFβ3, the band like accumulation of focal contacts disappeared and focal contacts became more diffusely arranged. This was accompanied by the TGFβ3 cells acquiring a more spread morphology. These results strongly indicate that TGFβ3 decreases the degree of cell attachment and promotes increased motility. This is in good agreement with the morphological changes observed using DIC microscopy, in which treatment with TGFβ3 resulted in cells acquiring a spread diameter up to a 3 fold that of untreated cells (Figs 1c, 1d). Our results not only show that TGFβ3 promoted increased spreading, but also show that TGFβ3 promoted an increase in cell replication rate after 24 and 48 hours (Fig 2). To further verify this finding, trypsinisation experiments were carried out to quantify TGFβ3 related changes in cell attachment.

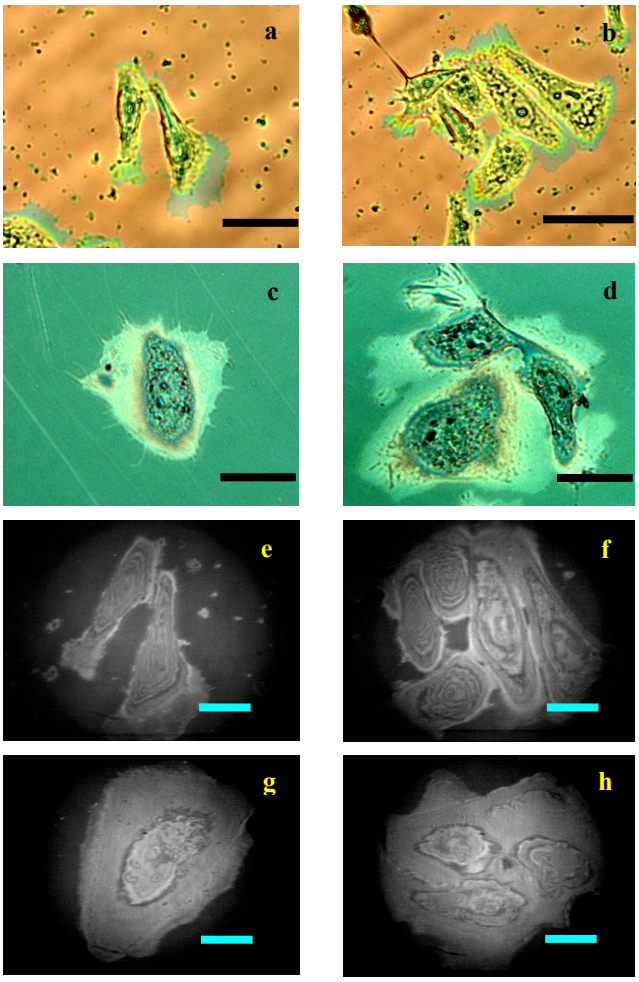

Fig. 1 (a) Image of individual and (b) group of fixed HaCaTs cells cultured without TGFβ3 on SPR gold coated substrate; (c) Image of individual and (d) group of fixed HaCaTs cells cultured with TGFβ3; Image taken with DIC microscope, objective x40 (*scale bar 35μm*); (e) the same individual cell as in (a) imaged with WSPR system; f) the same group of cell as in (b) imaged with WSPR system; (g) TGFβ3 treated single HaCaTs cell imaged with WSPR system; (h) TGFβ3 treated group of HaCaTs cell imaged with WSPR system (*scale bar 25μm*).

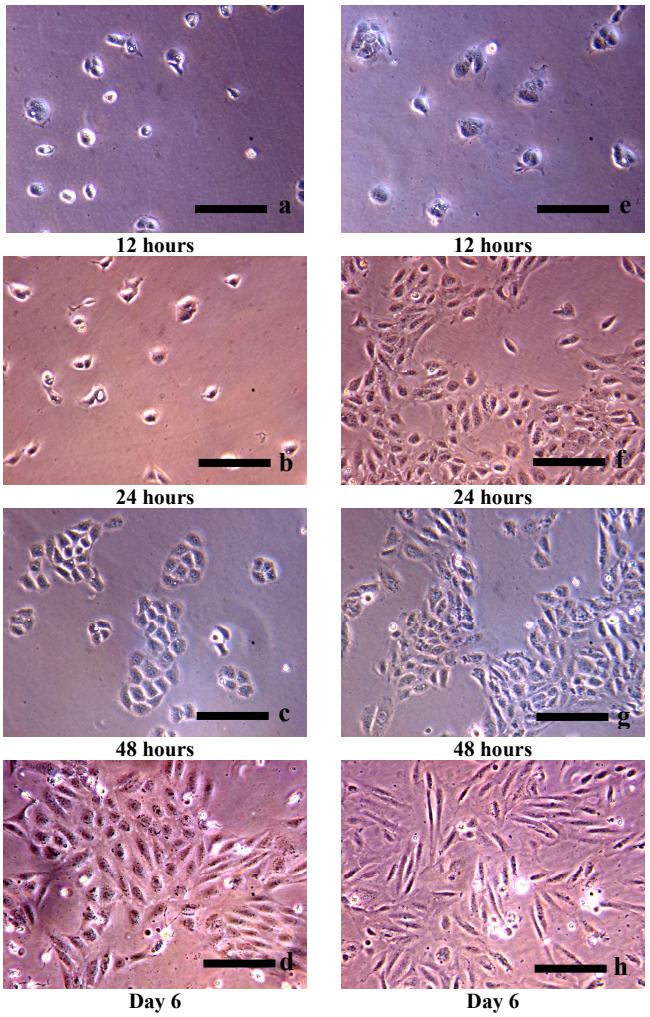

Fig. 2 (a) Image of HacaTs cell plated without the presence of TGFβ3- after 12 hours; (b) 24 hours; (c) 48 hours; (d) Confluence - Day 6, ready for Trypsinisation process; (e) Image of HacaTs cell plated with TGFβ3- after 12 hours; (f) 24 hours; (g) 48 hours; (h) Confluence - Day 6, ready for Trypsinisation process; Image taken with a standard phase contrast microscope, x10 objective (*scale bar 200μm*).

Trypsinisation Process: The trypsinisation experiments showed that HaCaTs cultured with TGFβ3 started to detach from the surface 5 seconds after application of trypsin (Figs 3a, 3b, 3c,) and completely detached by the second minute, (Figs 3d – 3g). On the other hand, a completely different response was recorded with the HaCaTs plated without TGFβ3. Their first detachment took place after 2 minutes (Figs 3k – 3n). More over, it is clear that even after seven minutes the cells were not completely detached and maintained a flattened phase dark appearance. These results confirm that application of TGFβ3 at 50ng/ml decreases the degree of cell surface attachment (Fig 3).

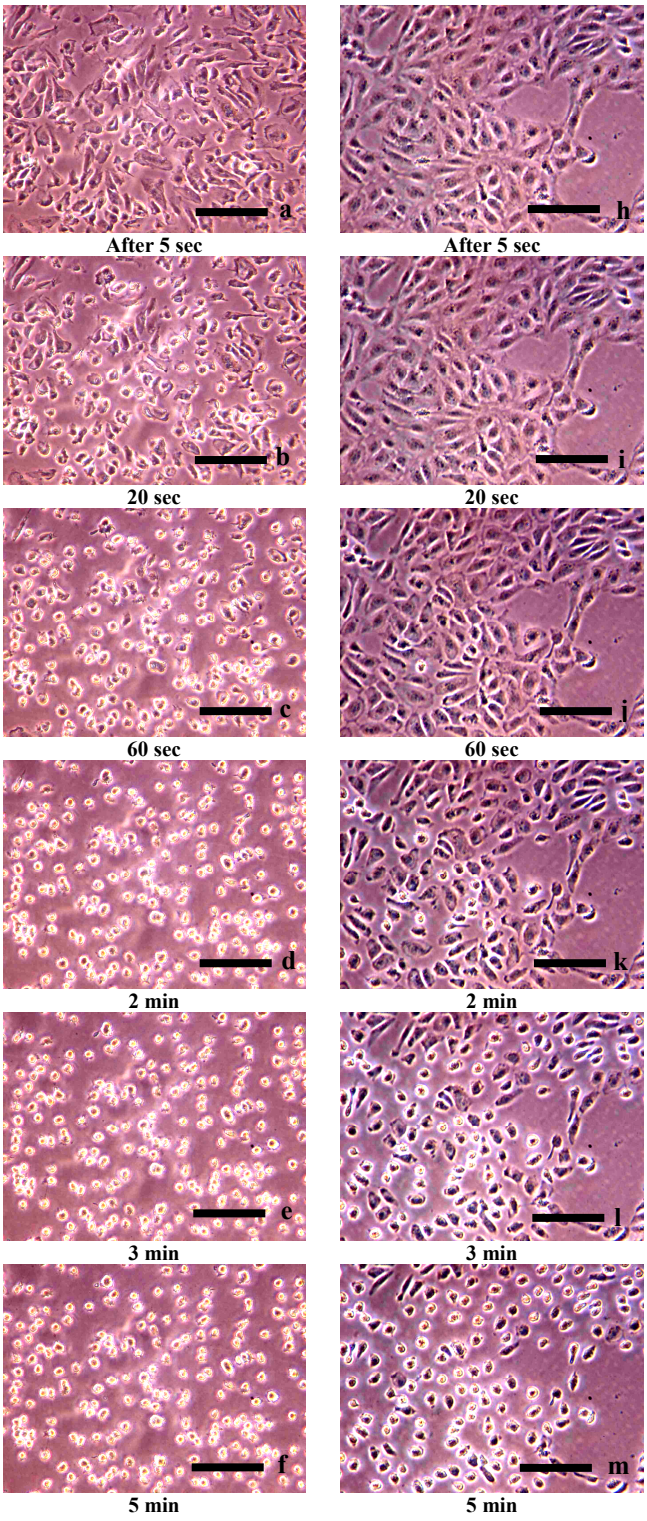

Fig. 3 Shows the sequence of images with time lapse captured for the trypsinisation process of HaCaTs cells plated with TGFβ3 (a, b, c, d, e, f, g) and without TGFβ3 (h, i, j, k, l, m, n) to show the detachment of HaCaTs cell from the culture flask. Image taken with a standard phase contrast microscope, x10 objective (*scale bar 200μm*).

IV. Conclusions

Traditionally cell surface contacts are investigated using immunostaining techniques, all of which require various step of staining and cell fixation. In this study we demonstrated that the new WSPR system can be used for studying cytokine induced changes in cell surface coupling/interactions at a high lateral resolution of less then a micron without the need for traditional immunostaining process. This finding represents a significant enhancement in cell imaging and will enable interrogation of cell surface interfacial interactions at a level that was previously unimaginable. This in turn will have significant implications in determining the functional characteristics of novel surface chemistries developed for use in wound healing systems.

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